Resistance of RNA-mediated TGS to HC-Pro, a viral suppressor of PTGS, suggests alternative pathways for dsRNA processing

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In plants, double-stranded (ds) RNA that is degraded to small (sm) RNAs that are ~23 nucleotides in length can trigger the degradation of homologous RNAs in the cytoplasm (posttranscriptional gene silencing or PTGS) and de novo methylation of homologous DNA in the nucleus [1]. PTGS is similar to quelling in fungi [2] and RNAi in animals [3]. RNA-directed DNA methylation (RdDM) can lead to transcriptional gene silencing (TGS) and the methylation of homologous target promoters if dsRNAs containing promoter sequences are involved [4]. HC-Pro is a plant viral suppressor of PTGS that acts by preventing the accumulation of smRNAs [5, 6] that provide the specificity determinant for homologous RNA degradation [7–10]. Here, we show that HC-Pro does not suppress TGS induced by promoter dsRNA. Moreover, the amount of promoter smRNAs is elevated 5-fold in the presence of HC-Pro, and target promoter methylation is slightly increased without a concomitant rise in the level of promoter dsRNA. The promoter dsRNA, which is not polyadenylated, failed to trigger substantial degradation of polyadenylated, single-stranded promoter RNA. The differential effects of HC-Pro on smRNA accumulation associated with dsRNA-mediated TGS and at least some cases of PTGS suggest that dsRNA processing can occur by alternative pathways, and they support the idea that RdDM is triggered by smRNAs.

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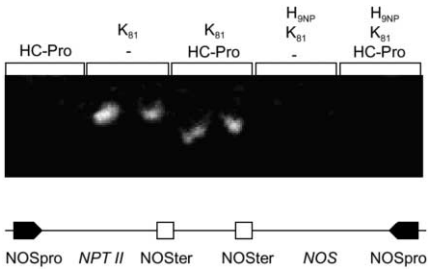
Results and discussion

Previous work has demonstrated that HC-Pro of tobacco etch virus does not suppress TGS in two different promoter homology-dependent gene-silencing systems, H_2 and 271 [11]. Because it was not clear whether silencing in these systems occurred through RNA silencing or homologous DNA pairing, we were interested in whether the H_{9NP} silencing locus, which induces TGS and the methylation of an unlinked target nopaline synthase promoter (NOSpro) via dsRNA that is transcribed from a NOSpro inverted repeat (IR) [4, 12], would be suppressed by HC-Pro. Crosses were made between a plant homozygous for a gene encoding HC-Pro and a plant doubly homozygous for the H_{9NP} silencing locus and a target locus, K_{81}, that contains a NOSpro-*NPTII* gene and an intact *NOS* gene [13, 14] (Figure 1). The F_1 plants were screened for the presence of nopaline. Analysis was focused on the *NOS* gene of the K_{81} locus because an *NPTII* gene is also present in the transgene complex containing the HC-Pro gene [11]. Nopaline was detected in plants containing the target K_{81} locus alone or in combination with the *HC-Pro* locus but was absent in K_{81}/H_{9NP} plants, owing to the synthesis of NOSpro dsRNA from the H_{9NP} locus, which silences the *NOS* gene and reduces nopaline levels at least 50-fold [12]. Nopaline continued to be undetectable in K_{81}/H_{9NP}/HC-Pro plants (Figure 1), demonstrating that TGS of the *NOS* gene triggered by NOSpro dsRNA was not significantly alleviated by HC-Pro.

The NOSpro of the *NOS* gene at the target K_{81} locus is normally not methylated (Figure 2a–c: K_{81}), and HC-Pro alone did not induce the methylation of target NOSpro or flanking sequences (Figure 2a–c: K_{81}, HC-Pro). The H_{9NP} silencing locus induces complete methylation of the K_{81} target NOSpro at both symmetrical (CpG and CpXpG; SacII) (Figure 2a, lanes S: K_{81} and H_{9NP}) and nonsymmetrical (CpX; DdeI) (Figure 2b, lanes D: K_{81} and H_{9NP}) cytosine residues within the region of homology between the NOSpro dsRNA and the NOSpro DNA. Methylation at Cs upstream of the NOSpro (Figure 2b, all genotypes) or in the *NOS*-coding region (Figure 2c, all genotypes) were not methylated in any genotype. Symmetrical and nonsymmetrical C methylation that is confined to the region of dsRNA-DNA homology is characteristic of RdDM [15]. This complete methylation persisted in the presence of HC-Pro (Figure 2a,b: K_{81}, H_{9NP} and HC-Pro). The only change observed was an approximately 50% increase in the level of partial methylation of a nonsymmetrical C at an NheI site at the border of NOSpro dsRNA-DNA homology in H_{9NP}/K_{81}/HC-Pro plants (Figure 2a, lanes N: compare H_{9NP}/K_{81} to H_{9NP}/K_{81}/HC-Pro).

Although dsRNA is required for the RdDM pathway [1, 4, 15], it is not known whether the intact dsRNA or smRNA degradation products provide the signal for homologous DNA methylation. The minimal DNA target size of

Figure 1



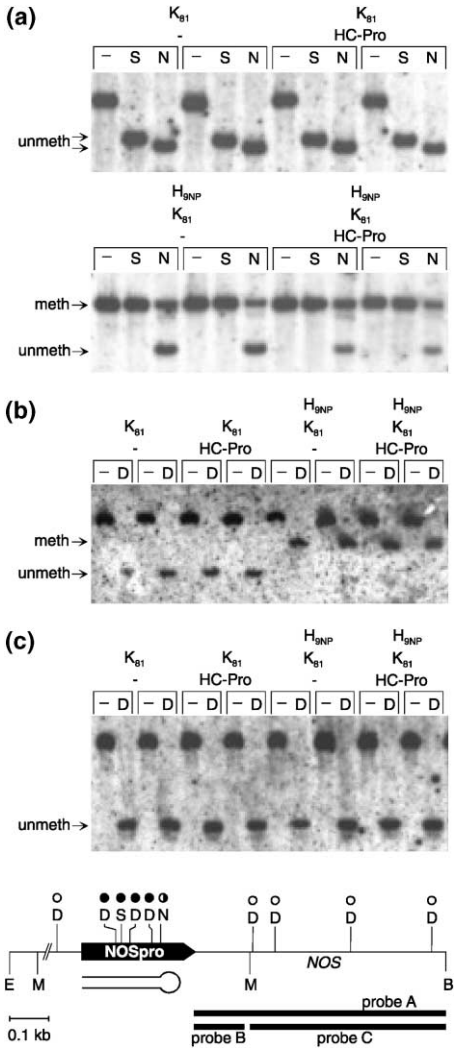
NOS gene silencing is not reversed by HC-Pro. The target K_{81} locus contains a neomycinphosphotransferase (*NPTII*) gene and a nopaline synthase (*NOS*) gene, each under the control of a NOS promoter (NOSpro) and NOS transcription terminator (NOSter) (bottom). The expression of the *NOS* gene in K_{81} and K_{81}/HC-Pro plants results in the accumulation of nopaline (white spot). *NOS* is repressed in the presence of the silencing H_{9NP} locus, which encodes NOSpro dsRNA (H_{9NP}/K_{81} lanes). HC-Pro does not reverse this silencing (H_{9NP}/K_{81}/HC-Pro lanes). The results from two plants of each genotype are shown. Nopaline was detected in leaf extracts using high voltage paper electrophoresis and phenanthrenequinone staining as described previously [14].

RdDM is 30 bp [16], hinting that the ~23-nt smRNAs direct the methylation of homologous DNA sequences. Because HC-Pro suppression of PTGS is accompanied by the loss of smRNAs [5, 6], this viral protein can potentially provide a tool to study RNA requirements for RdDM. Therefore, we tested K_{81}/H_{9NP}/HC-Pro plants for small NOSpro RNAs. Unexpectedly, instead of being reduced, the smRNAs were enriched approximately 5-fold in K_{81}/H_{9NP}/HC-Pro plants as compared to K_{81}/H_{9NP} plants (Figure 3a). Thus, in contrast to the suppressive effects of HC-Pro on smRNAs observed in PTGS systems tested so far [5, 6], smRNA accumulation associated with TGS was stimulated in plants expressing HC-Pro.

The enhanced accumulation of NOSpro smRNAs in the presence of HC-Pro could have been due to augmented transcription of NOSpro dsRNA, to increased cleavage of NOSpro dsRNA, or to improved stability of the smRNAs themselves. These hypotheses predict, respectively, that the amount of NOSpro dsRNA would either increase, decrease, or remain unchanged in HC-Pro-expressing plants. The latter hypothesis was supported by the observation that the amount of NOSpro dsRNA detectable in K_{81}/H_{9NP}/HC-Pro plants was roughly the same as that present in K_{81}/H_{9NP} plants (Figure 3b,) despite the 5-fold differences in smRNA levels. Therefore, the elevated quantity of NOSpro smRNAs observed in the presence of HC-Pro was probably due to the enhanced stabilization of these RNAs.

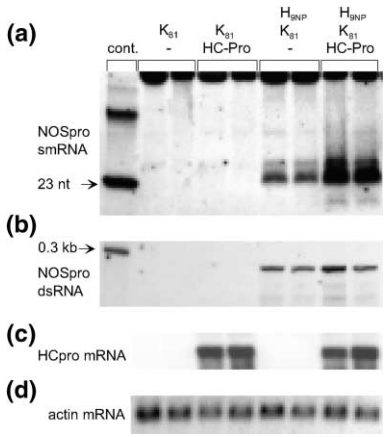
In an additional test of the silencing ability of NOSpro dsRNA, we introduced a transgene locus, H_{7NP}, which encodes a polyadenylated NOSpro single-stranded (ss)

Figure 2



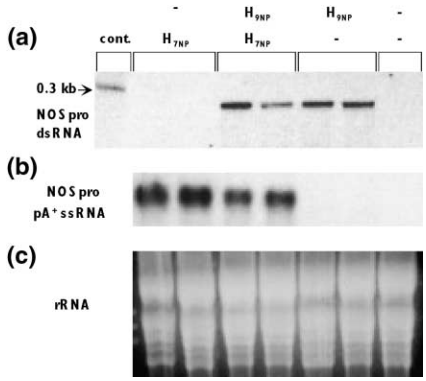
NOSpro methylation analysis. **(a)** The methylation-sensitive restriction enzymes SacII (S) and NheI (N) were used in conjunction with an EcoRV (E)/BamHI (B) double digest (minus lanes) to examine cytosine methylation in the NOSpro of the intact *NOS* gene. DdeI (D) together with either MvaI (M) or E/B was used to assess methylation in the **(b)** NOSpro and upstream region or the **(c)** *NOS*-coding region. Methylated and unmethylated fragments are indicated by the arrows. Complete, partial, and no methylation is indicated at each site on the gene map by the closed, half-open, and open circles, respectively. The SacII and DdeI sites in the NOSpro are fully methylated in the presence of the H_{9NP} locus (a,b). No methylation was observed at the (b) upstream DdeI site (methylation at this site would have led to the fragment size seen in the "minus" lanes) or in the (c) DdeI sites in the *NOS*-coding region. Partial methylation at the (a) NheI site increased approximately 50% in H_{9NP}/K_{81}/HC-Pro plants as compared to H_{9NP}/K_{81} plants. The black bars indicate the probes used for each blot. The results from two plants of each genotype are shown. The isolation of tobacco total DNA and Southern blotting were carried out as described previously [14]. SacII recognizes C*C*GCGG, NheI recognizes GCTAGC*, and DdeI recognizes C*TNAG. Asterisks indicate cytosine methylation known to inhibit enzyme activity. An increase in methylation at the NheI site was quantified by scanning autoradiograms with a Pharmacia Image Master System.

Figure 3



The enhanced accumulation of NOSpro smRNAs in the presence of HC-Pro. **(a)** smRNAs that are ~23-nt in length, which are generated by cleavage of NOSpro dsRNA transcribed from the *H_{9NP}* silencing locus (H_{9NP}/K_{81} lanes) increase approximately 5-fold in the presence of HC-Pro (H_{9NP}/K_{81}/HC-Pro lanes). The results from two plants of each genotype are shown. Similar results were obtained with both sense and antisense probes. A 23-nt size marker consisting of a NOSpro oligonuleotide is shown at the left. **(b)** The levels of NOSpro dsRNA in the H_{9NP}/K_{81} and H_{9NP}/K_{81}/HC-Pro plants differed by 50% or less. A NOSpro dsRNA control, which is 0.3 kb, was made by annealing separate sense and antisense NOSpro RNAs that were transcribed in vitro. **(c)** A Northern blot probed to demonstrate expression of HC-Pro. **(d)** A Northern blot probed with an actin probe to verify RNA quality and loading levels. The isolation of total RNA from tobacco leaves, gel electrophoresis, and Northern blot analysis using an actin probe were performed according to previously published procedures [12]. The methods used to isolate and analyze double-stranded RNAs and smRNAs are detailed in [4]. Differences in the amounts of dsRNAs and smRNAs were determined by scanning autoradiograms with a Pharmacia Image Master System.

Figure 4



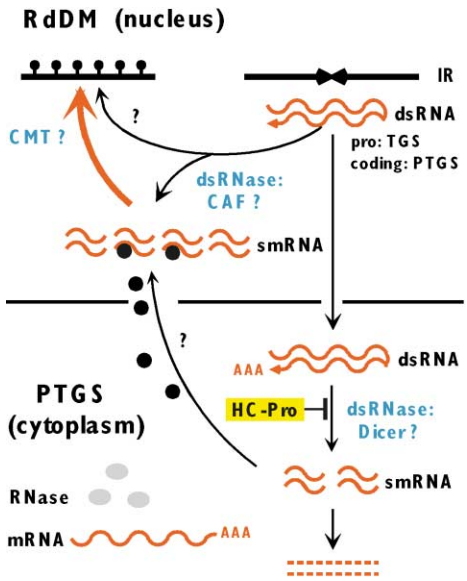
NOSpro dsRNA does not trigger PTGS of a polyadenylated NOSpro single-stranded RNA. **(a)** RNase protection experiments revealing the presence of NOSpro dsRNA encoded by the *H_{9NP}* locus. This RNA is not polyadenylated [4]. **(b)** Northern blot analysis demonstrating the presence of NOSpro ssRNA encoded by the *H7NP* locus. This RNA is polyadenylated [4]. Only partially decreased levels of NOSpro ssRNA were observed in the H_{9NP}/H_{7NP} plants tested, suggesting inefficient induction of PTGS by NOSpro dsRNA. **(c)** A stained gel showing rRNA bands to verify RNA quality and loading levels.

RNA [12]. Although this RNA is not translatable, other nontranslatable RNAs have been shown to be targets of PTGS [5, 17]. Only a partial diminishment of NOSpro ssRNA levels was observed in H_{9NP}/H_{7NP} plants (Figure 4), demonstrating that NOSpro dsRNA was unable to efficiently trigger PTGS of an mRNA-like NOSpro RNA.

These findings indicate that even though PTGS and TGS can each be initiated by dsRNA that is processed to smRNAs, alternative degradation pathways that are differentially affected by HC-Pro are involved. The choice of one or the other pathway might be based on qualitative or quantitative differences in dsRNAs derived from various sources. We believe that our data as a whole can be explained best by a model that posits separate nuclear or cytoplasmic compartmentalization of dsRNAs and processing proteins (Figure 5). HC-Pro proteins are encoded by RNA viruses that replicate exclusively in the cytoplasm, and they are not found in the nucleus except as components of inclusion bodies during advanced stages of pathogenesis [18]. The NOSpro dsRNA, which is not polyadenylated [4, 12], is transcribed from an IR in the nucleus, where we assume it is degraded to smRNAs because this degradation was not suppressed by HC-Pro. The nuclear localization of NOSpro dsRNA would also account for its inability to fully trigger PTGS of a polyadenylated NOSpro ssRNA that is presumably transported to the cytoplasm, the cellular compartment where PTGS predominantly occurs. DsRNAs triggering PTGS can be synthesized in the nucleus by transcribing through IRs, but they must be transported to the cytoplasm, which presumably occurs most efficiently with a polyA tract [19, 20]. DsRNA can also be produced in the cytoplasm by cellular or viral RNA-directed RNA polymerases [21, 22] acting on single-stranded RNA templates. HC-Pro only blocks the accumulation of smRNAs produced from a subset of dsRNAs, which we suggest are transported to, or synthesized in, the cytoplasm (Figure 5). Alternatively, the effects of HC-Pro on smRNA accumulation that we have observed might be restricted to those derived from dsRNAs synthesized from IRs. If so, smRNA accumulation associated with PTGS triggered by IR transgenes, similar to our TGS system, should be unaffected or even enhanced in the presence of HC-Pro.

While the cytoplasmic location of HC-Pro can account for its inability to block smRNA accumulation in the nucleus, an explanation is still required for the puzzling increase in the level of NOSpro smRNAs observed in HC-Pro-expressing plants. The fact that HC-Pro did not substantially alter the amount of dsRNA suggests that the increase in the amount of smRNAs could be accounted for by

Figure 5



A model to explain the differential effects of HC-Pro on RNA-mediated TGS (this study) and PTGS [5, 6]. RNA silencing in plants includes PTGS and RdDM. DsRNA (red, wavy lines), shown here as originating from the transcription of an inverted DNA repeat, plays a pivotal role in both types of silencing. HC-Pro, a cytoplasmic protein, suppresses the accumulation of guide smRNAs required for the sequence specificity of the RNase complex involved in PTGS (filled, gray circles), whereas its presence enhances the accumulation of smRNAs associated with TGS and RdDM of promoter sequences. We postulate that HC-Pro prevents a smRNA binding protein (filled, black circles) from stabilizing smRNAs in the cytoplasm, leading to further smRNA degradation to nucleotides (short dashes). The smRNA binding proteins are free to enter the nucleus and stabilize smRNAs in that compartment. Elevated levels of nuclear smRNAs might lead to increased DNA methylation caused by RdDM (thick, red arrow), although a direct role for unprocessed dsRNA in RdDM cannot be completely ruled out. Whether dsRNAs or ssRNAs are transported to the cytoplasm might depend in part on the presence of a polyA tail. Possible candidates for dsRNA processing to smRNAs include CARPEL FACTORY (CAF) [25] in the nucleus and a protein similar to Dicer [26] in the cytoplasm. RdDM could be directed by smRNAs that associate with the chromodomain [24] of chromomethylase (CMT) [22].

improved stability. Cytoplasmic HC-Pro might affect the nuclear accumulation of smRNAs by altering the binding activity of a protein that protects smRNAs from degradation. In this hypothesis, the smRNA binding protein would be present in limiting amounts and, unlike HC-Pro, would be able to shuttle between the cytoplasm and nucleus. HC-Pro might disrupt interactions between the smRNA binding protein and cytoplasmic smRNAs, freeing these proteins to enter the nucleus and stabilize smRNAs in that compartment. Unprotected smRNAs in the cytoplasm would be subject to enhanced breakdown and be unable to serve as guides for the RNase complex that degrades homologous mRNA (Figure 5). This model assumes that smRNAs are eventually degraded to nucleotides. The fact that the smRNAs and dsRNA are detectable simultaneously indicates that dsRNA degradation does not go to completion but is in steady state equilibrium with other steps.

A possible role for smRNAs in directing homologous DNA methylation is suggested by the increased methylation at an NheI site of the target NOSpro observed in K_{81}/H_{9NP}/HC-Pro plants, which have elevated smRNA levels. Further conclusions about smRNAs and RdDM are precluded in this study because methylation at both symmetrical and nonsymmetrical Cs was complete in the presence of the H_{9NP} silencing locus. However, it is attractive to consider that smRNAs derived from dsRNA cleavage are able to target not only RNA degradation in the cytoplasm but also homologous DNA methylation in the nucleus. A potential candidate for the DNA methyltransferase involved in RdDM is the chromomethylase [23]. This enzyme, found so far only in plants [24], could associate with a guide smRNA through the chromodomain, which can function as an RNA interaction module [25]. Processing of nuclear dsRNAs could be carried out by the product of the CARPEL FACTORY (*CAF*) gene [26], which encodes an *Arabidopsis* protein that is predicted to be in the nucleus and is related to *Drosophila* 'Dicer', an RNaseIII-like enzyme that cleaves dsRNA into 22-nt RNAs required for RNAi [27] (Figure 5).

There is increasing awareness that RNA can direct homologous DNA methylation and possibly target chromatin modifiers to specific regions of the genome [28]. RNA silencing at the posttranscriptional level plays important roles in host defenses to parasitic sequences and development in plants [1, 29, 30] and *Caenorhabditis elegans* [3, 31–33]. Whether the same holds for RNA-mediated TGS should be known soon from analyses of *Arabidopsis* mutants defective in this pathway.

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